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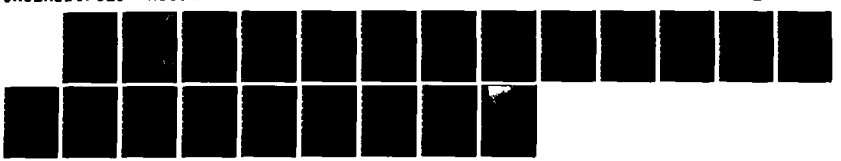

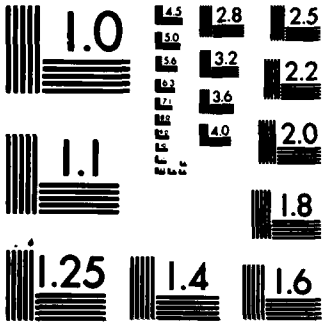


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# FACTORS GOVERNING THE SUBCELLULAR DISTRIBUTION OF INDIUM-111 IN HUMAN PLATELETS

by

J. L. COSTA, C. RUSHIN, J. J. VECCHIONE, AND C. R. VALERI

NAVAL BLOOD RESEARCH LABORATORY
BOSTON UNIVERSITY SCHOOL OF MEDICINE
615 ALBANY STREET
BOSTON, MA 02118

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The subcellular distribution of indium-111 (In-111), and the effect of the metabolic inhibitors rotenone and 2-deoxyglucose on its uptake, retention, and subcellular distribution, have been investigated in human platelets using techniques which permit the maintenance of dense body integrity during fractionation. As with chromium-51 (Cr-51), the In-111 label appears to be located principally in the cytosolic (soluble) fraction. Equilibrium dialysis studies suggest that only 10-20% of the In-111 is associated noncovalently with non-microsomal proteins. There appears to be a relationship between the

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metabolic pool of nucleotides and the uptake and retention of In-111, since incubation of platelets at 37 C with metabolic inhibitors prior to labeling with In-111 reduces the amount of label taken up when compared to platelets incubated at 22 C.

ABSTRACT

The subcellular distribution of indium-111 (In-111), and the effect of the metabolic inhibitors rotenone and 2-deoxyglucose on its uptake, retention, and subcellular distribution, have been investigated in human platelets using techniques which permit the maintenance of dense body integrity during fractionation. As with chromium-51 (Cr-51), the In-111 label appears to be located principally in the cytosolic (soluble) fraction. Equilibrium dialysis studies suggest that only 10-20% of the In-111 is associated non-covalently with non-microsomal proteins. There appears to be a relationship between the metabolic pool of nucleotides and the uptake and retention of In-111, since incubation of platelets at 37°C with metabolic inhibitors prior to labeling with In-111 reduces the amount of label taken up when compared to platelets incubated at 22°C.

The gamma-emitting isotope indium-111 (In-111) has been used by a number of investigators to label platelets.[1-4] Platelets can be tagged relatively rapidly and easily with the 8-hydroxyquinoline (oxine) complex of In-111, and the relatively short half-life and high gamma-emission efficiency of the isotope permit studies not possible with chromium-51 (Cr-51), the radioisotope used most commonly to label platelets for measurement of the platelet lifespan. In-111 itself can also be used to follow platelet turnover, since the kinetics of its disappearance from the platelet pool appear to parallel those observed when platelets are labelled with Cr-51.[5]

Molecules of Cr-51 associated with a platelet are believed to remain with a labelled platelet for its entire circulating lifespan, possibly because the Cr-51 is distributed throughout platelet cytoplasm rather than associated with any of the releasable subcellular organelles.[6] In-111 also appears to remain with a platelet for its entire lifespan, and has recently been reported to be bound non-covalently to proteins in the platelet cytosol.[4] We have investigated the association of In-111 with various subcellular fractions of human platelets, using a technique which (1) preserves the integrity of the dense bodies during fractionation and (2) distinguishes microsomes from the soluble cytosolic fraction. In addition, we have used equilibrium dialysis and treatment with metabolic poisons to explore the mechanisms responsible for the uptake and retention of In-111.

## MATERIALS AND METHODS

(1) Platelet isolation and labelling with In-111-oxine. For each experiment, 450 ml of whole blood was collected into the primary bag of a double pack (Fenwal Laboratories, Deerfield, IL) containing 67.5 ml of ACD anticoagulant (NIH formula A), using normal donors who gave no history of hematologic

disorders or drug ingestion for at least 10 days prior to collection. Platelet-rich plasma (PRP) was obtained by centrifuging the blood at 22° ± 2°C at 4500g for 2.5 minutes; PRP was acidified with 7% (V/V) ACD and then centrifuged at 4500g for 5 minutes. The platelet-poor plasma (PPP) was expressed, leaving approximately 30 ml of PPP in the bag; the bag was left undisturbed for 30 minutes at room temperature and the platelets were resuspended manually. The total number of platelets in these concentrates typically averaged $0.75 \times 10^{11}$. Platelet concentrate was washed free of plasma by adding 100 ml of 0.9% NaCl, centrifuging the mixture at 4500g for 5 minutes, and expressing the supernatant as completely as possible. 50 ml of 0.9% NaCl was added to the platelet pellet, which was left undisturbed for 10 minutes prior to manual resuspension. The saline-washed cells were incubated with 1 mCi of In-111-oxine (a complex of 8-hydroxyquinoline and In-111; Diagnostic Isotopes, Bloomfield, NJ) for 30 minutes in a 37°C water bath oscillating at 1 cycle per second. The incubated platelets were then washed by dilution (with 100 ml of PPP and 10% ACD), centrifugation (4500g x 5 minutes), and resuspension in 50 ml of PPP. The In-111-labelled platelet concentrate contained on the average $0.5 \times 10^{11}$ platelets (75% recovery). In one experiment resuspended platelets were simultaneously labelled with 1 mCi of Cr-51 (sodium chromate; E.R. Squibb and Sons, Princeton, NJ) by incubation as described above. Radioactivity was determined using a Model 1185 automatic gamma system (Tracor Analytic, Elk Grove Village, IL). In the experiment in which platelets were doubly labelled with In-111 and Cr-51, In-111 radioactivity was determined when Cr-51 crossover radioactivity contributed less than 1% of the total counts in the In-111 window, and Cr-51 radioactivity was measured after more than ten In-111 half-lives had elapsed (In-111 crossover radioactivity less than 1% of the counts in the Cr-51 window).

(2) Subcellular fractionation of In-111-labelled platelets. To avoid solubilization of the dense bodies during the cell disruption step, a modification of the procedure of Fukami et al.[7] was employed. 10 ml of the In-111-labelled concentrate was mixed with 10 ml of Tris-citrate buffer at pH 7.4 (116 mM NaCl, 4 mM KCl, 1.8 mM $NaH_2PO_4$, 1.1 mM $MgSO_4$, 25 mM Tris, and 10 mM citrate).[8] Platelets were centrifuged (4500g for 5 minutes) and resuspended in 25 ml of the same buffer containing 0.35% bovine serum albumin (BSA) (crystallized and lyophilized; Sigma Chemical Co., St. Louis, MO). Resuspended platelets were incubated for 20 minutes at 37°C with 20 μM rotenone and 5 mM 2-deoxy-D-glucose (Sigma Chemical Co.). Cells were then cooled at 22°C, mixed with 3 mg of Streptomyces griseus protease and 10 mg of ATP, incubated for 5 minutes at 22°C, and then mixed with 20 mg of soybean trypsin inhibitor (Sigma Chemical Co.). Platelets were centrifuged (1000g for 15 minutes), resuspended in 20 ml of 0.25 M sucrose containing 10 mM Tris and 1 mM EDTA at pH 7.4, and sonicated for 30 seconds at one-half maximum power (Branson Model W-350 Sonifier with a one-half inch tip). Subcellular fractionation of the sonicated platelets was carried out as described previously[7] by serial centrifugations at 1000g for 20 minutes (non-lysed platelets and large cellular debris), 12,000g for 20 minutes (dense bodies, lysosomes, alpha-granules, and mitochondria), and 100,000g for 60 minutes (microsomes). At each step, aliquots of the pellet and supernate were removed for measurement of the amount of radioactivity in each fraction. Protein remaining in the supernatant from the 100,000g spin (the cytosolic fraction) was removed by the addition of trichloracetic acid (TCA) (final concentration, 5%) and centrifugation at 38,000g for 20 minutes.

(3) Dialysis of the cytosolic fraction. 20 ml of the cytosolic fraction (supernatant from the 100,000g spin) before or after TCA precipitation were

placed in dialysis tubing permeable to molecules with a molecular weight of less than 12,000 daltons. The bag and contents were then dialyzed against 1 liter of 10 mM Tris buffer, without EDTA, pH 7.4, at 4°C for either 24 or 36 hours. In some cases, 0.1% bovine serum albumin (Sigma Chemical Co.) was added to the Tris buffer to determine whether the presence of a non-specific binding protein would alter the dialysis behavior of In-111.

(4) Effects of metabolic inhibitors on retention and subcellular location of In-111. 50 ml of saline-washed platelets were prepared as described above and divided into 3 equal portions. Portion 1 was incubated at 22°C for 20 minutes, portion 2 at 37°C for 20 minutes, and portion 3 at 37°C for 20 minutes with the metabolic inhibitors 20 μM rotenone and 5 mM 2-deoxy-D-glucose. To check on the extent of metabolic inhibition,[9] a 0.2 ml aliquot of platelets from each portion was observed for aggregation in response to 40 μM ADP. 250 μCi of In-111-oxine was then added to each portion and all were incubated for 30 minutes at 37°C. Portions were diluted with 20 ml of PPP plus 10% ACD, centrifuged (4500g x 5 minutes), and resuspended in 10 ml of PPP. The percent uptake was calculated by dividing the amount of In-111 associated with pellets following resuspension by the amount of In-111 present in the cells plus that in the supernatant prior to resuspension (i.e., the total amount added to the bag prior to labeling). These once-washed platelets were diluted with 10 ml of Tris-citrate buffer, pH 7.4 (see above), centrifuged (4500g x 5 minutes), and resuspended in 25 ml of the same buffer containing 0.35% bovine serum albumin. Aliquots were again taken for measurement of the percent uptake. The twice-washed platelets were then incubated as described above with rotenone and 2-deoxyglucose and subjected to subcellular fractionation.

## RESULTS

From 9% to 16% of the In-111 present in intact platelets was associated with mitochondria, α-granules, lysosomes, and dense bodies, and a similar

percentage with the microsomal membranes (Table 1). From 57% to 70% of the label remained in the organelle- and membrane-free cytosolic fraction, and precipitation of the soluble protein with TCA did not remove a significant amount of the label from this fraction. In platelets labelled with both In-111 and Cr-51, the distribution of the two labels between various fractions was essentially identical (Table 2).

To explore the possibility that cytosolic In-111 might be associated with soluble components of molecular weight greater than 12,000 daltons, a series of dialysis experiments was carried out (Table 3). Given the relative volumes of the dialysis bag and buffer, complete equilibration of In-111 across the dialysis membrane would have left 2% of the total label inside the bag and 98% in the buffer. Since 20% of the In-111 remained inside the dialysis bag after 24 hours at 4°C, some material present in the cytosolic fraction apparently was capable of binding In-111. The affinity of this material for In-111 appeared to be significantly higher than that of bovine serum albumin, because addition of 0.1% bovine serum albumin to the dialysis buffer also permitted more than 20% of the In-111 to remain inside the bag. After 36 hours of dialysis with or without added bovine serum albumin, approximately 11% of the In-111 remained inside the dialysis bag. The dialysis system thus may have been approaching equilibrium relatively slowly, or may have been subject to a time-dependent alteration in the affinity of the cytosolic binding material for In-111. When protein in the cytosolic fraction was removed by TCA precipitation prior to dialysis for 24 hours at 4°C, less than 4% of the In-111 remained in the dialysis bag.

To examine the relationship between the metabolic integrity of platelets and the uptake and retention of In-111, we measured the amount of cell-associated In-111 when cells were incubated with metabolic poisons immediately prior to labeling with In-111 (Table 4). In Experiment 1, the In-111-labeled

platelets resuspended in PPP (see Materials and Methods) were placed on a shaker at room temperature for two hours prior to measurement of the cell-associated In-111. In Experiment 2, cell-associated In-111 was measured immediately after resuspension in PPP. After incubation for 30 minutes at 22°C, labeling, and one wash, platelets aggregated normally with ADP, and 59% of the total In-111 added remained with the platelets. A second wash removed on the average 15% of this cell-associated In-111. Cells incubated for 30 minutes at 37°C aggregated normally in response to ADP, retained approximately 50% of the added In-111 following one wash, and lost 21% of this label after a second wash. Cells incubated for 30 minutes at 37°C with 2-deoxy-D-glucose and rotenone failed to aggregate with ADP. These cells retained on the average 29% of their In-111 after one wash, and lost on the average 45% of their label after a second wash. Despite the reduced uptake and retention of In-111 in metabolically poisoned cells, the same percentage of the cell-associated label was found in the cytosolic fraction as in control cells (Table 5).

## DISCUSSION

The subcellular localization of In-111 in human platelets was evaluated recently by Hudson et al.[4] These workers reported that following nitrogen-bomb disruption and fractionation in sucrose gradients according to the procedure of Broekman et al.,[10] from 65-73% of the platelet-associated In-111 was located in a "cytosol" fraction. Based on this finding, and on the pattern of elution of In-111 in the "cytosol" fraction from a Sephadex G-100 column, Hudson et al.[4] concluded that the In-111 labeled human platelets was associated predominantly with soluble cytoplasmic components.

There are two methodologic concerns which may interfere with acceptance of the validity of this conclusion:[10] (a) nitrogen-bomb lysis apparently disrupts platelet dense bodies, resulting in solubilization of their contents,

and (b) the "cytosol" fraction was not further centrifuged to separate any membrane vesicles or fragments from more soluble components. Thus "cytosolic" In-111 observed by Hudson et al.[4] may have come from dense bodies, or may have been associated with or sequestered inside a microsomal type of membrane vesicle.

To address these concerns, we repeated the subcellular fractionation utilizing a technique reported to preserve the integrity of the dense bodies during the isolation process.[7] In addition, we compared the amount of In-111 associated with membranes in the microsomal fraction by centrifuging at 100,000xg for 60 minutes and measuring In-111 in the pellet (membranes) and supernatant.

With both these technical manipulations, our data confirm the preferential association of the intra-platelet In-111 with the soluble (cytoplasmic) fraction. The In-111 appears not to be bound covalently to protein or lipid in this fraction (i.e., is not precipitable with TCA), and is essentially identical in location to Cr-51 when platelets are labeled simultaneously with the two isotopes. Nevertheless, in our dialysis experiments without EDTA, more than 80% of the cytosolic In-111 was removed following dialysis for 24 hours at 4°C, and more than 88% after 36 hours at 4°C. Since bovine serum albumin was no more effective than buffer alone, only a relatively small proportion of the cytosolic In-111 may be bound strongly by cytoplasmic proteins with a molecular mass of more than 12,000 daltons. When compared to the extensive association of In-111 with components of 25,000 and 46,000 daltons reported by Hudson et al.,[4] the low degree of association seen here may be due to an alteration or breakdown of binding sites during the 24-hour dialysis period at 4°C, or to the fact that our preparation was relatively free of membrane material.

The suggestion that binding to cytoplasmic proteins may not play a major role in the uptake and retention of In-111 by human platelets is supported by our data on the effects of metabolic poisons on In-111 disposition. Incubation of washed platelets for 30 minutes at 37°C with inhibitors of both oxidative phosphorylation (rotenone) and anaerobic glycolysis (2-deoxy-D-glucose) dramatically decreases the adenylate energy change, a measure of the total high-energy phosphate bonds available to participate in cellular metabolism, but does not result in appreciable leakage of cytoplasmic proteins.[9] The effects of this treatment on the uptake of In-111 might therefore be expected to result from the loss of cellular energy stores, rather than from a reduction in cytoplasmic protein. Furthermore, the effects do not appear to be due to loss of intracellular phosphate groups potentially capable of binding In-111. First, under normal circumstances, essentially all the cytoplasmic ATP is complexed to magnesium ions.[11,12] A relatively small amount of In-111 is retained by cells (less than 0.01% of the amount of metabolic ATP present, or an estimated cytoplasmic concentration of 0.3 μM), and this amount would be unlikely to compete successfully with magnesium ions (estimated cytoplasmic concentration, 5 mM) for binding to ATP. Second, although metabolic poisoning dephosphorylates more than 70% of the metabolic ATP to produce hypoxanthine and inosine (which are rapidly lost from the cell), the phosphorus moieties appear to be retained as inorganic phosphate and fructose 1,6-diphosphate.[13,14]

The decreased platelet labeling with In-111 seen after metabolic poisoning, and even after incubation at 37°C, suggests that a similar alteration may occur when In-111 is used to label platelets which have been stored for 72 hours. Platelets stored for 72 hours have been reported to display impaired metabolic capacity, including a decreased adenylate energy change and metabolic ATP pool.[15] Neveretheless, the platelet In-111 after poisoning apparently remains

cytoplasmic in location, and is no more likely to be lost from the cell during washing than is In-111 in the control cells. These observations, coupled with the relatively close parallels between the intracellular disposition of Cr-51 and In-111 in normal cells, support the conclusion that labeling with In-111 may provide a useful altlernative to Cr-51 labeling for both fresh and stored platelets.

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Table 1. Localization of Indium-111 in Subcellular Fractions of Indium-111-Oxine Labelled Platelet Concentrate

| Experiment | Percentage of Total Platelet Label in Subcellular Fractions | | | | |
|---|---|---|---|---|---|
| | Unlysed Cells, Large Debris | Vesicles and Mitochondria | Microsomal Fraction | Cytosolic Fraction | |
| | | | | Untreated | After Protein Precipitation |
| J | 13.9% | 9.2% | 6.6% | 70.3% | -- |
| K | 14.8% | 16.3% | 10.4% | 58.5% | 58.0% |
| L | 8.4% | 11.2% | 10.0% | 70.4% | 69.8% |
| M | 19.4% | 24.1%* | | 56.5% | -- |
| Mean ± one S.D. | 14.1 ± 4.5% | 12.2 ± 3.7% | 9.0 ± 2.1% | 63.9 ± 7.5% | 63.9 ± 8.3% |

* This value is for the vesicles and mitochondria plus the microsomal fraction, and is not included in the means.

Table 2. Localization of Indium-111 and Chromium-51 in Subcellular Fractions of Indim-111-Oxine and Chromium-51 Double-Labeled Platelet Concentrate

| Label Examined | Percentage of Total Platelet Label in Subcellular Fractions | | | | |
|---|---|---|---|---|---|
| | Unlysed Cells, Large Debris | Vesicles and Mitochondria | Microsomal Fraction | Cytosolic Fraction | |
| | | | | Untreated | After Protein Precipitation |
| Indium-111 | 8.4% | 11.2% | 10.0% | 70.4% | 69.8% |
| Chromium-51 | 5.2% | 7.6% | 6.5% | 80.7% | 75.8% |

Table 3. Effects of Dialysis on Indium-111 Present in the Supernatant Fraction of Sonicated Platelets

| Dialysis Procedure | Percent of In-111 in Each Compartment | |
|---|---|---|
| | Dialysis Bag | Buffer |
| Ratio expected from relative volumes of bag and dialysis buffer | 2% | 98% |
| 1. Untreated supernatant | | |
| 24 hours at 4°C with | | |
| Tris buffer | 20.2% | 76.8% |
| Tris + 0.1% BSA | 23.2% | 80.5% |
| 36 hours at 4°C with | | |
| Tris buffer | 11.7% | 83.6% |
| Tris + 0.1% BSA | 10.9% | 86.1% |
| 2. Protein-free supernatant | | |
| 24 hours at 4°C with | | |
| Tris buffer | 3.5% | 95.7% |

Table 4. Effects of Varying Incubation Conditions Prior to In-111 Labeling on the Retention of the In-111 During Post-Labeling Washes

| Treatment Prior To Addition of In-111 | Experiment Number | Percent of Total In-111 Associated With Platelets | |
|---|---|---|---|
| | | After 1 Wash (Resuspend in PPP) | After 2 Washes (Resuspend in PPP and then Buffer) |
| Incubate 30 minutes at 22°C | 1 | 59.0% | 52.6% |
| | 2 | 59.4% | 48.1% |
| Incubate 30 minutes at 37°C | 1 | 49.3% | 40.8% |
| | 2 | 49.7% | 36.1% |
| Incubate 30 minutes at 37°C with metabolic poisons | 1 | 17.6% | 8.1% |
| | 2 | 40.3% | 23.9% |

Table 5. Comparison of the Localization of Indium-111 in Subcellular Fractions of Control Platelets and Platelets Incubated with Metabolic Poisons Prior to Labeling with Indium-111

| Treatment Prior To Addition of Indium-111 | Percentage of Total Platelet Label in Subcellular Fractions | | |
|---|---|---|---|
| | Unlysed Cells, Large Debris | Vesicles Mitochonrida, and Microsomes | Cytosolic Fraction |
| Incubate 30 minutes at 37°C | 20.1% | 23.3% | 56.6% |
| Incubate 30 minutes at 37°C with metabolic poisons | 15.6% | 27.4% | 57.0% |